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Effect of Gibberellic Acid and Actinomycin D on the Formation and Distribution of Rough Endoplasmic Reticulum in Barley Aleurone Cells¹

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Abstract:

Analysis of structural changes in barley aleurone cells during germination or following incubation of isolated layers in gibberellic acid with or without actinomycin D revealed extensive development of rough endoplasmic reticulum. Following the assembly of stacked rough endoplasmic reticulum, vesiculation occurred mainly in basal regions of the cell, resulting in a polar distribution of rough endoplasmic reticulum vesicles. It is postulated that these vesicles are involved in protein secretion, because smooth vesicles, derived from the rough endoplasmic reticulum, apparently become appressed to the plasma membrane. The increased α -amylase in the ambient medium

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and in cell homogenates correlated directly with formation and subsequent vesiculation of the rough endoplasmic reticulum. Furthermore, when cells were treated with actinomycin D and gibberellic acid, α -amylase synthesis was inhibited by 45% and secretion by 63%. These cells were characterized cytologically by large areas of disarrayed segments of fragmented rough endoplasmic reticulum, corresponding to a high intracellular level of α -amylase. In addition, small lipid bodies common to the segmented regions of rough endoplasmic reticulum were surrounded by fine fibrous material, short segments of rough endoplasmic reticulum, and free ribosomes, suggesting that actinomycin D had interfered with development and organization of rough endoplasmic reticulum.

Changes in cellular organelles and endomembranes have been reported for aleurone cells of germinating grains (3) and isolated layers treated for various lengths of time with GA (1, 13, 15, 28). However, the specific sequence of events leading to synthesis and secretion of hydrolases has not been adequately established. This is partly due to the difficulty of fixation of aleurone cells. Fine structural and biochemical studies have shown that a specific secretory granule, such as the zymogen granule, is not formed in aleurone cells (12-14).

In a recent biochemical analysis of aleurone cells during the lag phase of α -amylase synthesis an increase of polysome formation and membrane synthesis was reported (4-7). In addition, two enzymes of the cytidine diphosphatecholine pathway, which is involved in lecithin biosynthesis, increased within 2 hours after GA application (11). We find in the present study that synthesis and secretion of α -amylase is associated with an increase in RER,² which is arranged into large stacks of interconnected cisternae. Secretion of α -amylase apparently depends on the vesiculation of the assembled RER, a process that begins at the terminals of the cisternae facing the plasma membrane. Thus, the critical involvement of formation and modification of endomembranes becomes apparent in the GA-mediated responses.

Materials and Methods

Aleurone layers in groups of 10, prepared from surface-sterilized, imbibed half-grains of barley (*Hordeum vulgare* L. var

²Abbreviations: RER: rough endoplasmic reticulum; AcD: actinomycin D; ER: endoplasmic reticulum.

Himalaya), were incubated on a shaker in 2 ml of buffer containing 1 mM sodium acetate, pH 4.8, and 100 mM CaCl_2 (1). The 1.35 μM GA (Merck) and/or 100 $\mu\text{g/ml}$ actinomycin D (Calbiochem) were added to the incubation mixture as required. α -Amylase activity was measured in the medium and in whole cell homogenates at various periods after incubation at 25°C. Tissue homogenates were prepared by grinding rinsed and blotted aleurone layers in 2 ml of 165 mM HEPES buffer, pH 7.5, containing 0.4 M sucrose, 60 mM KCl, 10 mM MgCl_2 , 10 mM EDTA, and 10 mM dithiothreitol. Enzyme activity was measured in the supernatant following centrifugation for 10 minutes at 2000g. Protein in the medium and homogenate supernatant was determined according to the method of Lowry et al. (19), using freshly prepared bovine serum albumin (Calbiochem) as the standard.

Electron Microscopy. Aleurone cells were fixed for 4 hour at 5°C with one of the following fixatives: (a) Karnovsky's glutaraldehyde-paraformaldehyde in 0.05 M cacodylate buffer, pH 7.4 (16); (b) 2.5% glutaraldehyde in 0.05 M cacodylate or phosphate buffer, pH 7.4; (c) 4% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.4; (d) 4% paraformaldehyde in 0.05 M collidine, pH 7.4, containing 0.3 M or 0.4 M sucrose and 10 mM CaCl_2 . After washing in fixative buffer (0.1 M), tissue sections of about 50 μ were prepared from thin tissue strips with an Oxford Vibratome system (Oxford Equipment, San Mateo, California). These sections were postosmicated for 2 to 4 hours at 5°C in 2% osmium tetroxide buffered to pH 7.2 with 0.05 M buffer (cacodylate, phosphate, or collidine). Osmicated tissue previously fixed with glutaraldehyde in cacodylate buffer was washed in several changes of 0.1 M sodium cacodylate buffer, pH 7.4, followed by at least four rinses in distilled water for 1 to 2 hours before staining with 2% aqueous uranyl acetate for 14 hours at 5°C (30). It was important that both the osmium tetroxide and buffer were removed completely from the cells before staining; otherwise precipitates often formed inside the cells. Tissue sections from different fixations were rinsed several times in water after osmication, dehydrated in a graded series of alcohol and propylene oxide, and embedded in either Luft's Epon 312 mixture (20) or Spurr's low viscosity resin mixture (27). Epon-Araldite mixtures generally gave unsatisfactory results, presumably because of slow diffusion of the more viscous araldite resin and exchange of propylene oxide. Tissue segments were kept rotating in 25% and 50% resin mixtures for at least 6 hours and in 75% for 12 to

14 hours. This was followed by five changes of 100% resin over the next 24 to 48 hours. Tissue sections were blotted on filter paper before being placed in fresh 100% resin in flat embedding molds. The molds were placed in a vacuum oven overnight before polymerization at 65°C without a vacuum for 24 to 48 hours for Epon 312 or 12 hours for Spurr's mixture.

Embedded tissue was sectioned with a diamond knife on a LKB Ultratome III and blue-grey to silver sections were picked up on 150 mesh, carbon-coated grids. The sections were stained for 10 minutes to 1 hour in saturated uranyl acetate in 50% ethanol or 2% aqueous uranyl acetate followed by 5 minutes in lead citrate (26) before viewing in a Siemens Elmiskope I at an accelerating voltage of 80 kv or a RCA EMU 4 at 50 kv.

Tissue Preparation. Preparation of aleurone cells for electron microscopy proved difficult, owing to the large amount of stored lipid and protein and thick cell walls. As reserve lipid and protein were metabolized during germination, however, good fixation of aleurone cells was obtained with procedures used routinely for other tissues. In the present study, half strength Karnovsky fixative gave adequate fixation for aleurone layers after 22 hours of incubation in GA, while formaldehyde alone with sucrose was very good for aleurone tissue from shorter periods of incubation in GA. Prolonged fixation in glutaraldehyde alone, while providing relatively good fixation of aleurone layers, resulted in swelling of the RER. Formaldehyde, on the other hand, caused considerable swelling of mitochondria and RER vesicles unless sucrose was present. Thus, the tonicity of the fixative used was very important in preventing artifactual changes in vesicles

Table I. Effect of Gibberellic Acid and Actinomycin D on α -Amylase Production

Ten aleurone layers were incubated 22 hr with or without GA (1.35 μ M) and/or actinomycin D. All numbers are the average of four separate experiments.

Treatment	α -Amylase			α -Amylase Released into Medium
	Medium	Homogenate	Total	
		μ g		%
–GA	32.0	23.4	55.4	57.7
+GA	124.0	34.8	158.8	78.2
+GA + 100 μ g/ml Ac D	32.4	55.0	87.4	37.1
–GA + 100 μ g/ml Ac D	20.5	28.1	48.6	42.3

and organelles. For generally good fixation, 4% formaldehyde in 0.05 M collidine containing 0.3 M sucrose and 10 mM CaCl_2 was the fixative of choice, providing good preservation of membranes and organelles which stood out more readily in the less dense cytoplasmic matrix.

RESULTS

Aleurone Cells of Germinating Grains. Aleurone cells adjacent to the scutellum from grains germinated for 36 hours contained numerous lipid bodies and aleurone grains which compressed and partially distorted mitochondria, plastids, and microbodies (Figure 1). Large stacks of rough membranes prominent in peripheral regions of the cell consisted of interconnecting sheets of endoplasmic reticulum in parallel array. Surface sections of this RER revealed the presence of many ribosomes in polysome configuration. Membrane-bound polysomes were not, however, limited to the peripheral stacks of RER but were also prominent beside the nucleus, in association with small segments of RER (Figure 1). Enhanced and extensive vesiculation of the RER was observed after 48 hours of imbibition, at which time cells in the same region close to the scutellum were highly vacuolate and contained many rough-surfaced vesicles free in the cytoplasm.

Isolated Aleurone Layers Incubated with GA. Aleurone cells treated with GA for various periods of time from 9 to 66 hours underwent changes similar to those observed in aleurone cells from germinating grains. Development of stacked RER, which varied somewhat in time of appearance in different experiments, was first observed at 12 hours after GA treatment. At approximately 16 hours the stacked RER was found fragmented into spherical to ellipsoidal vesicles which appeared more numerous near the cell wall (Figure 2). These RER vesicles averaged $0.36 \times 0.14 \mu$ in size. The increased number of RER vesicles along the plasma membrane coincided with α -amylase secretion (Table I). In control tissue not treated with GA very little RER was observed, and only a small amount of α -amylase was found (Table I). Polar distribution of RER vesicles in the cytoplasm along the basal and radial walls was observed in cross sections of GA-treated aleurone tissue (Figures 3-6). RER vesicles were present in the apical region of these cells, but much less evident (Figures 3, 4, 6).

The cytoplasm-plasma membrane interface, which contained many rough-surfaced vesicles, also included smooth vesicles, free ribosomes, dictyosomes with associated vesicles, and mitochondria (Figures 3-5). Apparent fusion of smooth vesicles with the plasma membrane was observed as shown in the inset of Figure 5. Smooth vesicles were pleomorphic and contained a dense, fibrous matrix, similar in appearance to that in RER vesicles (inset, Figure 5). In contrast, Golgi vesicles, which also appeared to fuse with the plasma membrane, were more uniform in shape and electron-translucent (Figure 4). Periodic indentations along the plasma membrane were also common in these cells (Figure 5). Frequently, the plasma membrane appeared to be composed of several overlapping layers (Figure 4).

Disorganization of the cell wall which was observed in areas adjacent to regions of cytoplasm occupied by rough and smooth vesicles (Figures 3-5) resulted probably from localized digestion of cell wall components along the plasma membrane to form narrow electron-lucid channels (arrows in Figures 3-5). Larger channels of irregular outline were more obvious in outer regions of the cell wall, resulting from more extensive disorganization of cell wall material (Figures 3-5). These interconnecting channels, which were different from the plasmodesmata, but often associated with the latter, were also seen in

the radial walls. Some of these digested areas appeared to connect with channels in the basal walls, especially at the junctions of three or four cells. The degree of cell wall digestion observed after 16 hours of GA treatment increased progressively from the pericarp to the endosperm (Figures 3-5).

Aleurone cells treated up to 66 hours with GA underwent progressive vacuolation and diminution of RER vesicles, corresponding to lower levels of extractable α -amylase. Lysosomal-like structures, however, were not observed in these cells, even though the aleurone tissue became extremely soft.

GA- and Ac D-treated Cells. Light microscopic examination of cells

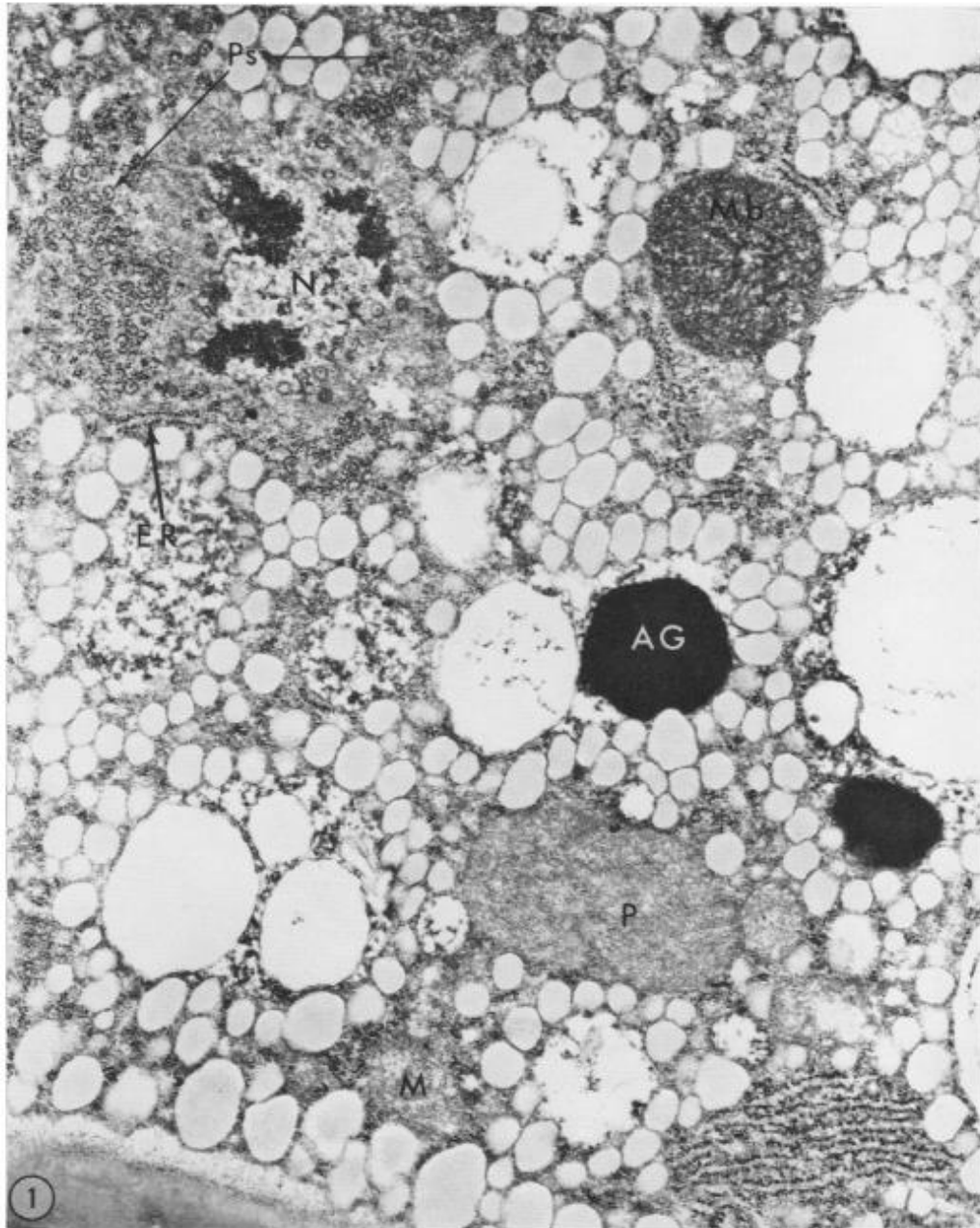


Figure 1. Aleurone cell after 36 hours of germination. Lipid bodies (LB) and Aleurone grains (AG) are most prominent in contrast to other organelles including mitochondria (M), plastids (P), and microbodies (Mb). The close association of polysomes (Ps) to nuclear pores can be seen along the periphery of the nucleus (N). X 23,100.

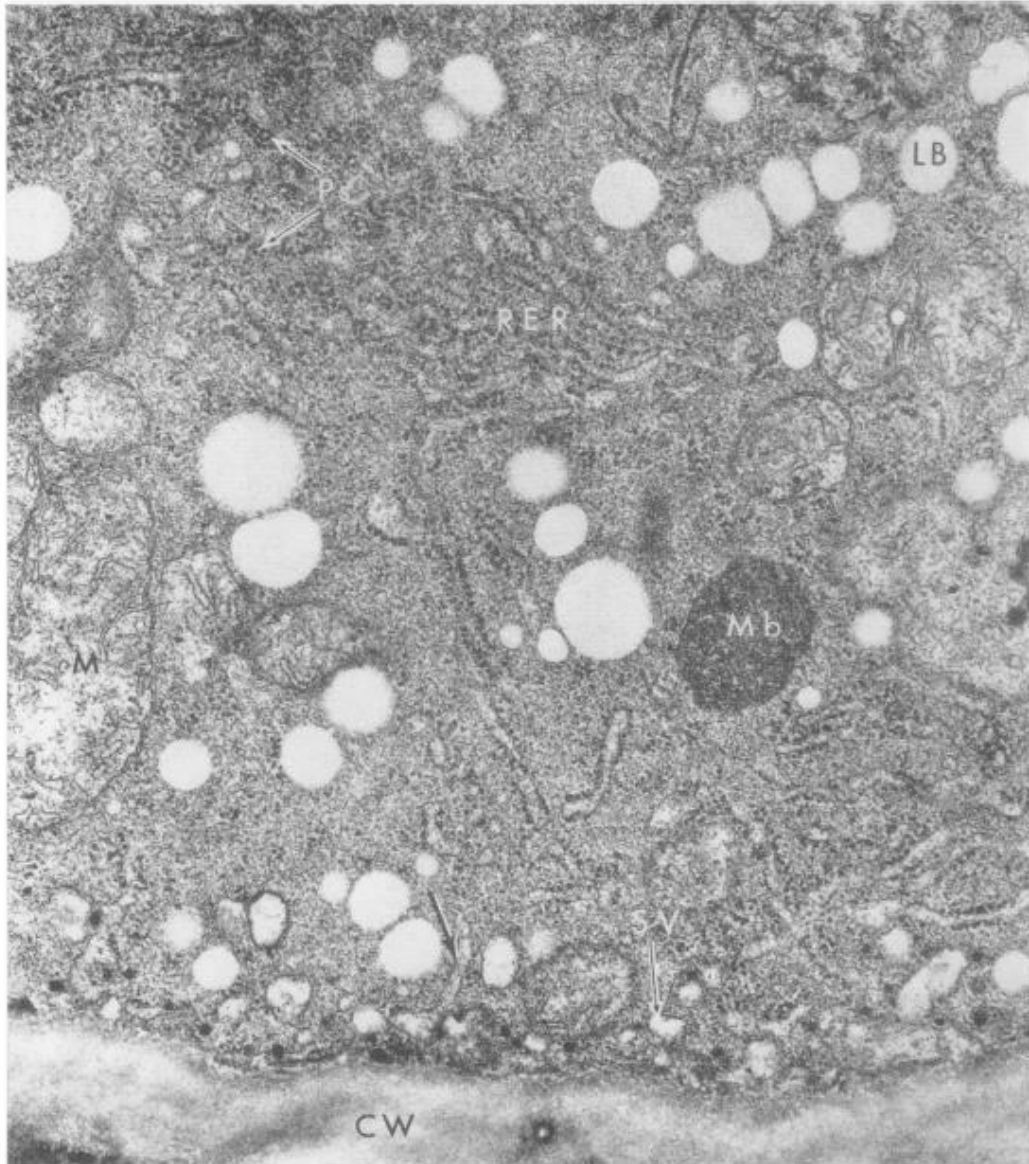


Figure. 2. Portion of an aleurone cell treated with GA for 16 hours RER containing polysomes (Ps) appears to be dissociating into small vesicles. Some cisternae appear devoid of ribosomes (arrow) as do smooth vesicles (SV) along the plasma membrane. Mitochondria (M) are quite prominent as are lipid bodies (LB) with only a single microbody (Mb) evident. Cell wall (CW). X 49,900.

from aleurone layers treated for 22 hours with Ac D and GA revealed large accumulations of basophilic material sometimes adjacent to the nucleus. Such accumulations were not observed in cells treated with GA alone. A single nucleolus devoid of vacuoles was found in nuclei of

cells treated with Ac D and GA. The nuclei also differed in appearance to cells treated with GA alone, owing to the presence of compact chromatin. The basophilic regions were identified under the electron microscope as RER, which, in contrast to the large uninterrupted arrays of interconnected cisternae in cells treated with GA alone, possessed definite fragmented cisternae (Figure 7). Such aggregated segments of fragmented RER were found only in cells treated with Ac D and GA. In one of the four experiments with GA, and Ac D, the RER was neither segmented nor stacked to any extent. More importantly there was no vesiculation of the RER in these cells. Polysomes were present on the surface of the fragmented RER (Figure 8), but not adjacent to the nuclear pores (Figures 7, 8). Also, small lipid bodies present within the RER area were generally surrounded by fine fibrous material, free ribosomes, and short segments of RER (Figure 8). This fibrous material was often attached to short segments of RER (arrows, Figure 8). Such a lipid-RER relationship was not observed along the

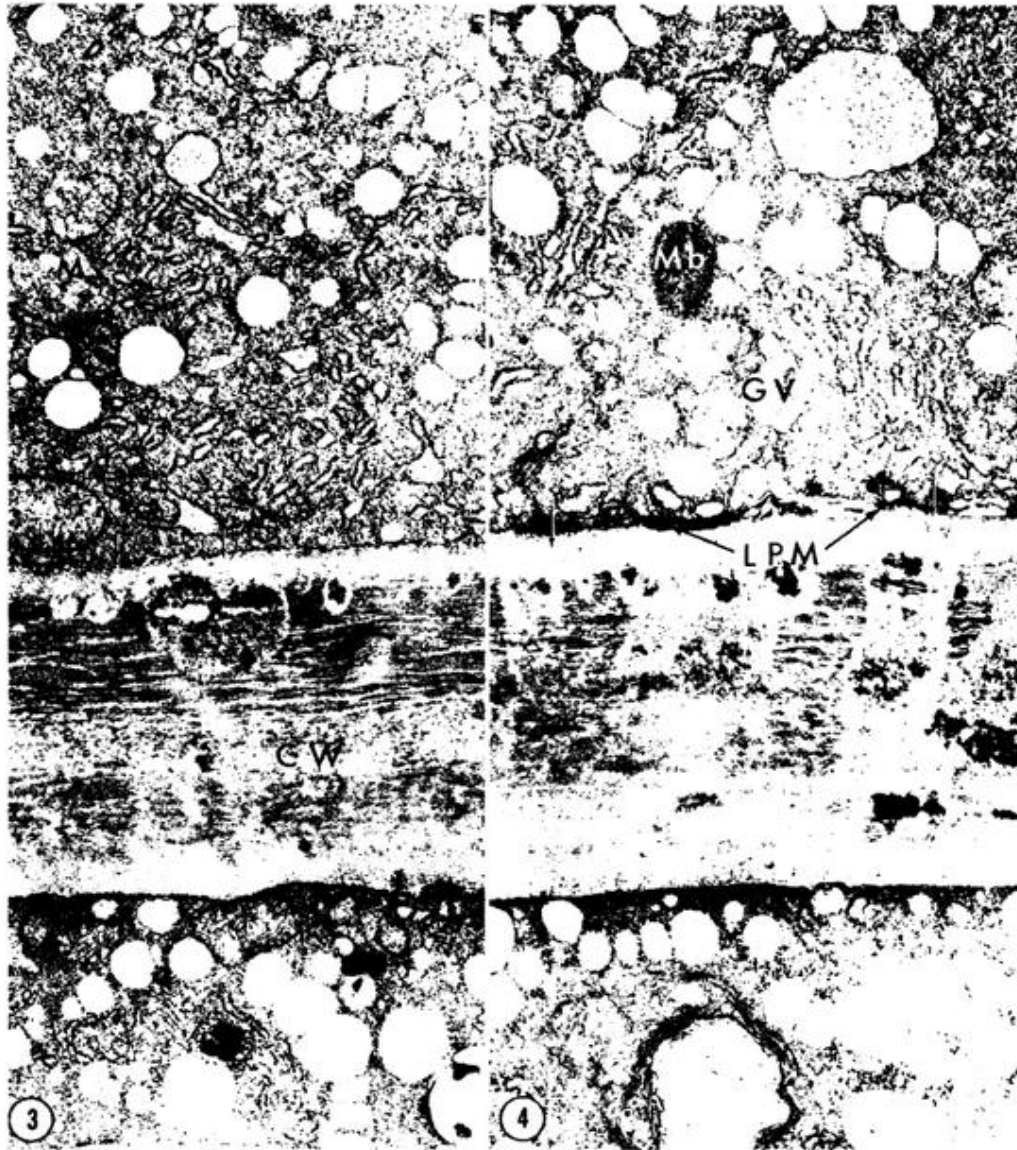


Figure 3. Cross sectional view of two aleurone cells situated near the fruit wall from aleurone tissue treated with GA for 16 hours. Numerous RER vesicles abound along the plasma membrane in the basal portion of the upper cell, but not in the apical region of the lower cell. Small clear channels in the cell wall opposite the plasma membrane (arrows) connect with large disarrayed regions further in the cell wall (CW). X 15,500.

Figure 4. Portions of two cells from the same section as Figure 3, but further away from the fruit wall. Golgi (G) and Golgi vesicles (GV) are evident and some of the latter appear to have fused with the plasma membrane, resulting in a laminated appearance (LPM). To the right of the Golgi vesicles are numerous RER vesicles; some nearest the plasma membrane contain fewer ribosomes and appear smooth.

Disorganization of cell wall material is extensive and extends from the plasma membrane via narrow channels (arrows). X 18,830.

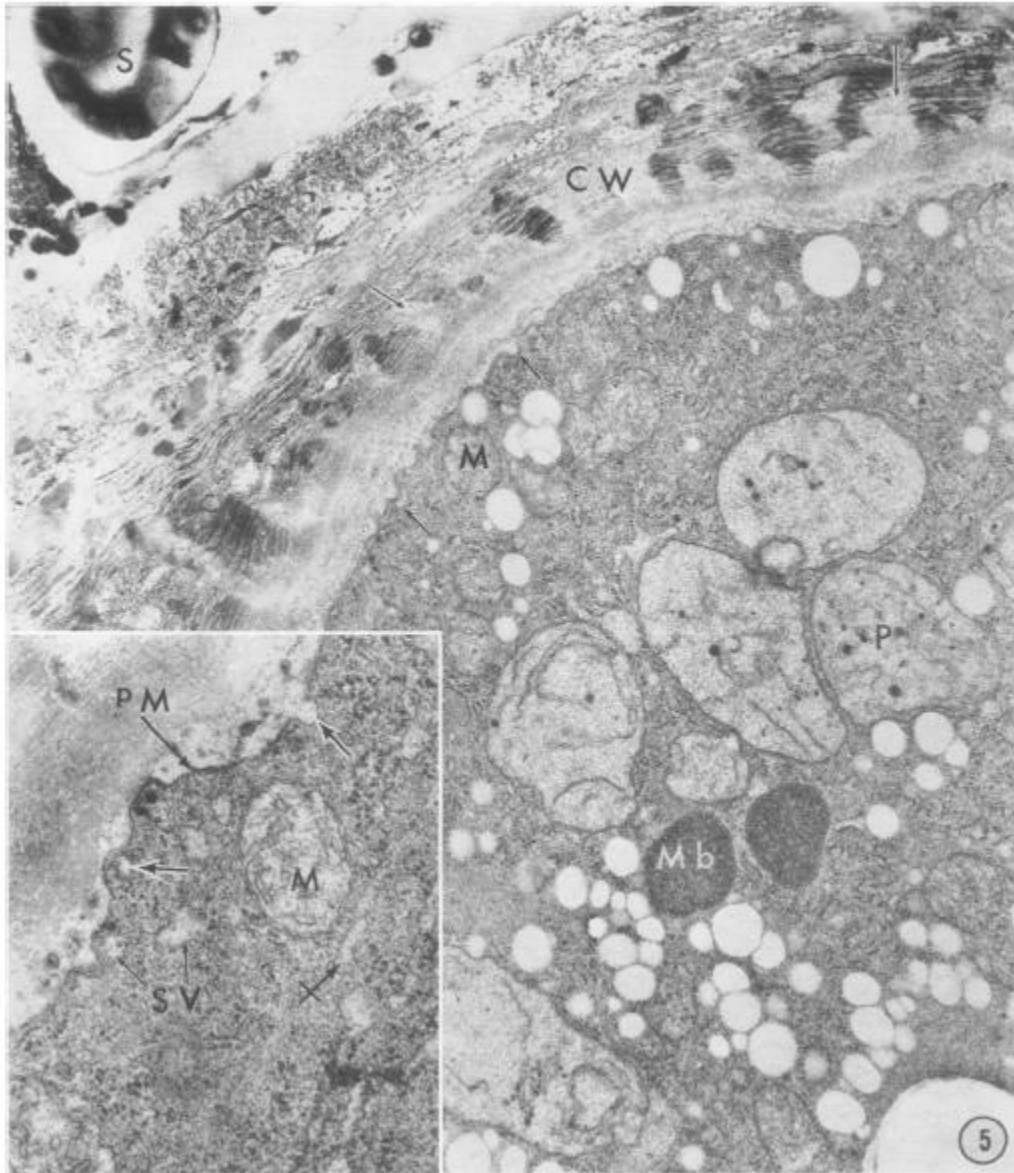


Figure 5. Bottom cell in series with Figures 3 and 4, being adjacent to the starchy endosperm (S). Disorganization of the cell wall is very pronounced (large arrows). The plasma membrane (PM) has a very undulated appearance (small arrows). Opposite one of these undulations there is a smooth vesicle (lower small arrow). Numerous mitochondria (M) subtend the plasma membrane. Plastids (P); microbodies (Mb). X 13,500. Inset: Apparent fusion of smooth vesicles (SV) with the plasma membrane appears to be occurring in this portion of a cell treated for 16 hours with GA (arrows).

RER has large regions devoid of ribosomes, appearing in some areas to be completely smooth (crossed arrow). X 29,400.

periphery of RER areas where the lipid bodies were much larger and not in association with cisternae (Figures 7, 8) nor in any cell treated with GA alone (Figure 2). Occasionally a mitochondrion (Figure 7) or a microbody was found within an area of segmented RER. Organelles other than the RER did not appear to be altered in their fine structure by Ac D. However, some retention of lipid bodies and storage protein was observed, and dilated cisternae (32) appeared to increase in number and often could be found appressed and partially enveloped by a microbody (33).

Enzyme Activity. The amount of α -amylase released into the incubation medium and present in the homogenate of intact cells was



Figure 6. Low magnification of an aleurone cell facing the endosperm (bottom of figure) after 16 hours of GA. Numerous RER vesicles abound in the basal portion of the cell and are seen in detail in the inset. The apical region contains mainly laminated RER (upper inset). X 6,830; upper inset: X 12,850; lower inset: X 11,000.

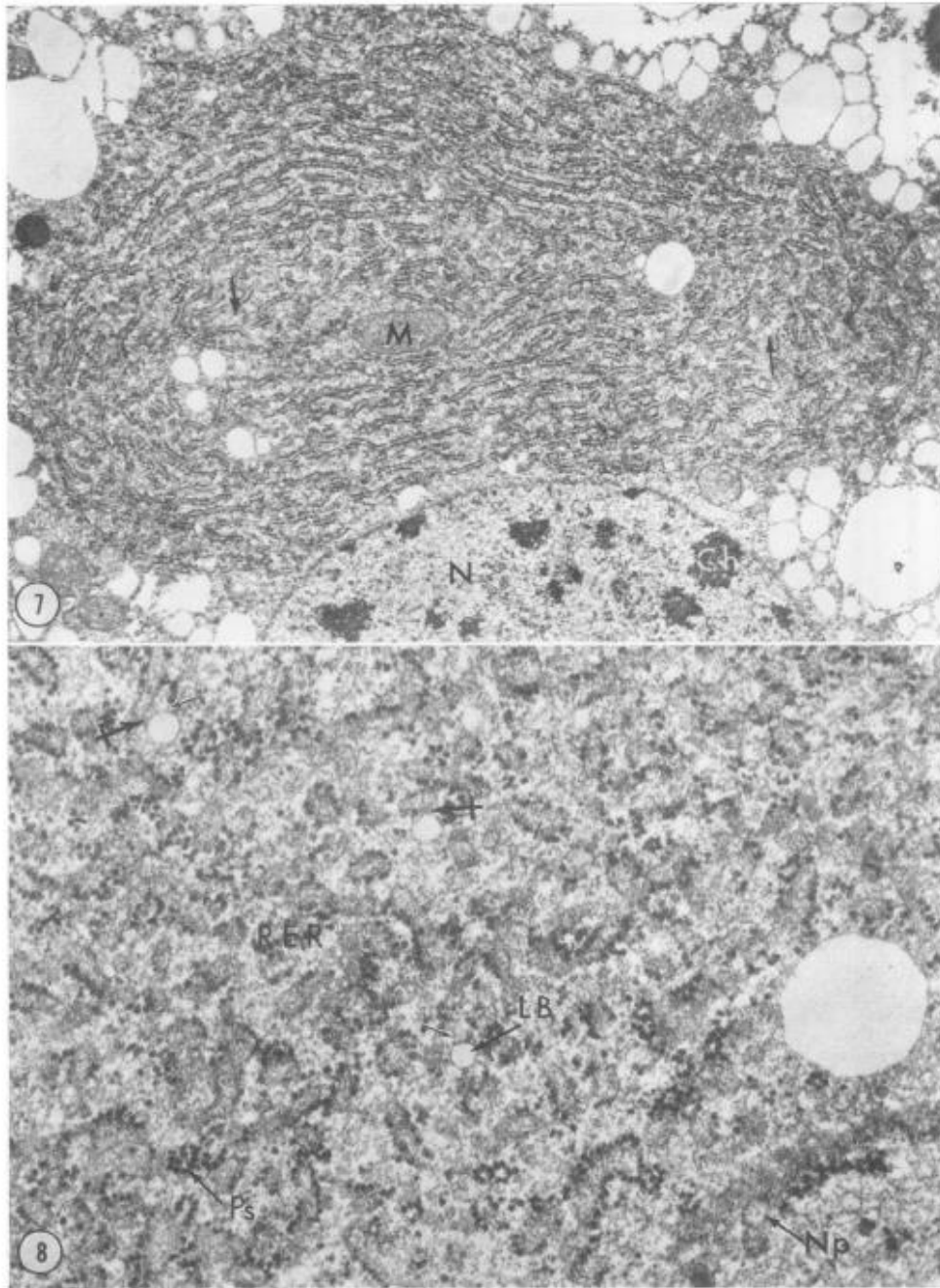


FIG. 7. Portion of a cell treated with GA and Ac D showing partial lamination of RER segments contained in a large mass along the nucleus (N). Chromatin (CH) is present in small patches which is not true for GA-treated cells. A single mitochondrion (M) is present within the RER mass which also contains numerous small lipid bodies (arrows). X 12,600.

FIG. 8. Enlargement of RER illustrating the predominance of segmented endoplasmic reticulum surrounding small lipid bodies (LB). Fibrous extensions from the LB often connect with short segments of RER (crossed arrows). The nuclear pores and surrounding area are free of any ribosomes or polysomes (Ps) that are present on segmented RER. X 50,000.

measured in order to correlate fine structural changes with enzyme synthesis and secretion. As shown in Table I, Ac D inhibited synthesis as well as secretion of α -amylase (see also Reference 2). The total amount of α -amylase synthesized by aleurone cells treated with GA and Ac D was only 55% of those treated with GA alone. Cells treated with GA secreted 78% of α -amylase in contrast to only 37% when treated with Ac D and GA. Hence, a larger amount of α -amylase was retained in aleurone cells as a result of Ac D action. The accumulation of segmented rough cisternae within cells treated with Ac D and GA correlated directly with the retention and also the limited synthesis of α -amylase, implicating the RER as a functional component involved in secreting α -amylase.

Discussion

The earliest and most significant GA-mediated effect in aleurone cells which can be observed in the electron microscope was the formation of RER cisternae and the subsequent aggregation of these cisternae into large stacks of laminated RER. These events were identical in aleurone cells from germinating caryopses and in isolated layers treated with GA. Measuring incorporation of ^{14}C -choline into membranes, Evins and Varner (6) were able to show that ER synthesis increased within 4 hours after GA application and polysome formation within 3 to 4 hours (4, 5, 7). Maximal level of polysome formation was attained 10 to 11 hours after GA treatment, which coincided in time with the appearance of massive stacks of RER. Membrane synthesis, which is stimulated by GA (17), is common to all these events. It is therefore of interest that the activity of two enzymes involved in lecithin biosynthesis in aleurone cells is enhanced by GA (11).

Hormone-stimulated RER formation apparently is a general phenomenon occurring in both animal and plant cells. Tata has shown an increase in RER during precocious initiation of enzyme synthesis in thyroid hormone-induced tadpole metamorphosis (29). Hormone-induced RER formation has been observed frequently in protein-

secreting cells (23, 24, 29). In these cells nascent protein is supplied to distinct secretory vesicles or granules by passage through extension of the smooth ER to the Golgi apparatus, followed by condensation in secretory vesicles, or by passage directly from the smooth ER to the plasma membrane (22). The latter membrane flow system may operate in barley aleurone tissue which can be regarded as a functional-ephemeral holocrine gland.

During the period of maximal secretion of α -amylase, which was measured as a convenient marker enzyme known to be induced by GA (8, 35), an accumulation of RER vesicles was observed along the plasma membrane (Figures 3-6). The unequal distribution of these vesicles between basal and apical regions of aleurone cells (Figure 6) suggests their involvement in a selective mode of secretion. The number of vesicles along the basal cell wall and to some extent along the lateral walls was always greater than in comparable apical regions of the cell. Indication of fusion of smooth vesicles with the plasma membrane was observed only in basal and lateral regions of these cells, where rough vesicles, free ribosomes, and mitochondria were abundant. The plasma membrane appeared undulated in those regions. In addition, cytoplasmic vesicles which fuse with the plasma membrane require membrane transformation before fusion may occur (21, 22).

The assumption that smooth vesicles derived from the RER have a secretory function is further supported by experiments with Ac D-treated aleurone cells. Ac D application resulted in impaired lamination and vesiculation of the RER, which correlated directly with the impaired synthesis and secretion of α -amylase. The percentage of α -amylase secreted was below that of the control (Table I). RER vesicles were not observed in cells treated with Ac D and GA for 22 hours, but numerous short segments of RER accumulated within the cells (Figures 7, 8). Therefore, it seems likely that secretion of enzymes from aleurone cells involves the RER directly, following distinct biochemical and morphological transformations that lead to assembly and dispersal of RER vesicles as a GA-mediated process. Examination of intracellular distribution of hydrolases in GA-treated aleurone cells demonstrated the presence of some α -amylase and glucanase in particulate cell fractions, but the highest enzyme activity was found in the supernatant (14). This suggests the possibility that, if any hydrolases

were in RER vesicles of barley aleurone cells, they were probably released into the supernatant during cell homogenization (9).

Microbodies were not found to be closely associated with lipid bodies (34) as was first observed in castor bean endosperm (32), nor did they increase noticeably in number during depletion of stored lipids. Therefore, gluconeogenesis may not be an important process in barley aleurone cells during germination, but microbodies may be involved in supply precursors for membrane synthesis (33), or a site for nitrate metabolism (18).

In the region of vesicle fusion with the plasma membrane, mitochondria were always numerous (Figures 3-8) and showed strong cytochrome oxidase staining with DAB (34). This distribution of the mitochondria may be important for enzyme secretion, which was shown to be highly energy dependent in both plant (31) and animal (10) cells.

The effect of Ac D on the formation of RER requires further comment. While Ac D has been shown to inhibit uridine incorporation into RNA in aleurone cells (1, 2), it also prevents the incorporation of ³P-labeled phospholipids into membranes of chick fibroblast (25). The close association of small lipid bodies with the cisternae of the RER in cells treated with GA and Ac D suggests the inhibition of specific steps in the GA-enhanced membrane synthesis. It is important that Ac D also inhibited the GA-enhanced activity of phosphorylcholine-cytidyl and phosphorylcholine-glyceride transferases (11). The formation of short segments of rough cisternae may indicate that Ac D inhibited ER membrane synthesis at specific loci.

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